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Leishmania tropica in Rock Hyraxes (*Procavia capensis*) in a Focus of Human Cutaneous Leishmaniasis

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Abstract. Cutaneous leishmaniasis, caused by *Leishmania tropica*, has recently emerged in urban and rural foci of central and northern Israel, and constitutes a major public health concern. Rock hyraxes (*Procavia capensis*), the suspected natural reservoir, were trapped in the cutaneous leishmaniasis urban focus of Maale Adumim in central Israel and evaluated for *L. tropica* infection by real-time kinetoplast DNA (kDNA) polymerase chain reaction (PCR) and serology. Real-time PCR on blood and computerized western blot serology analysis was positive for *L. tropica* in 58% and 80%, respectively, of the hyraxes tested. Phylogenetic analysis of the ribosomal internal transcribed spacer 1 region indicated that similar genotypes were present in humans and hyraxes from the same habitat. The high rates of infection and exposure to *L. tropica* among hyraxes supports their involvement in the transmission cycle of this parasite, and their potential role as a reservoir for human disease.

INTRODUCTION

Three species of *Leishmania* are endemic in Israel. *Leishmania major* and *L. tropica* cause cutaneous leishmaniasis (CL), whereas *L. infantum* is the causative agent of visceral leishmaniasis.[1] There has been a marked increase in the incidence of CL caused by *L. tropica* during recent years in Israel, Jordan, and the Palestinian Authority, and infection has emerged in new urban and rural foci.[2–9] Between 2000 and 2002 over 60 cases of human CL, most caused by *L. tropica,* were reported from near the Sea of Galilee in Northern Israel.[10] More recently a focus of *L. tropica* CL emerged in Maale Adumim (MA), a city located in the Judean Desert in central Israel. Reports of the Israel Ministry of Health in the Jerusalem district cited 54 and 73 new *L. tropica* CL cases in MA during 2004 and 2005, respectively. Most of the CL patients in MA reside in houses bordering a central gorge inhabited by hyraxes.[9] These numbers reflect a sharp increase in the annual incidence from 2 cases per 100,000 residents between 1999 and 2003 to 214 cases per 100,000 residents in 2004.[11]

In laboratory experiments, sand fly vectors of *L. tropica* readily bite hyraxes infecting a large proportion of the animals.[12] A previous study in northern Israel indicated that 10% of the hyraxes sampled in a rural CL focus were positive for *L. tropica* by conventional polymerase chain reaction (PCR).[7] Hyraxes live 8–9 years in nature,[13] and thus comprise an efficient reservoir for carrying infections into the next transmission season.[14] They can sustain experimental infection by *L. tropica* and are infective to sand flies while showing no clinical signs.[12,14] Thus, hyraxes fulfill, to a large extent, the criteria necessary to be reservoir hosts for *L. tropica*.[14,15]

The purpose of the present study was to evaluate *L. tropica* exposure and infection rates among hyraxes living in an urban focus of disease in central Israel using quantitative real-time PCR and serology, and to compare the genetic relatedness of parasites isolated from hyraxes to those found in humans and sand flies.

MATERIALS AND METHODS

Study area. The study was carried out in the city of MA, which is located in the Judean Desert at an altitude of 350 meters above sea level approximately 5 km east of Jerusalem. The human cases in MA were clustered in two neighborhoods located adjacent to slopes and caves inhabited by rock hyraxes.[11]

Hyraxes. Rock hyraxes were trapped in March 2005 using cage-traps as a part of an epidemiological investigation of the human CL focus in MA conducted by the Israeli Nature Reserves and Parks Authority. Animals were anesthetized (10 mg/kg intramuscularly Ketamine-HCl, Ketaset, Fort Dodge Animal Health, Fort Dodge, Iowa) using a pole syringe, physically examined, weighed, and blood samples were collected. The study that concerned animals was conducted adhering to the Hebrew University's guidelines for animal husbandry and use of animals in research. Blood in ethylenediamidinetetraacetic acid (EDTA) tubes was used for DNA extraction. Blood samples for serology were separated using centrifugation and kept at −20°C for further analysis. Experimentally infected (EI) hyrax samples were used for standardization of the western blot assay, as well as PCR and real-time PCR tests. EI samples included hyrax no. 1, a hyrax inoculated by subcutaneous injection in the nose with 10^7 *L. tropica* promastigotes supplemented with promastigote secretory gel; and hyrax no. 2, infected by sand fly bite.[12] Both hyraxes tested positive by PCR of nose skin biopsies for *L. tropica* infection. In addition, blood samples from 5 young hyraxes born in captivity in a non-endemic area of Israel were used as negative controls.

Human patients. Parasites were cultured for routine diagnosis from residents of MA (N = 8), Tiberias, a city located on the Sea of Galilee (Kinneret) in the South Kinneret (SK) region in northern Israel (N = 3), and Karkom, a village in the North Kinneret (NK) region in northern Israel (N = 1). These residents presented with cutaneous lesions and were referred to the Dermatology Department of the Hadassah Hospital, Jerusalem, during 2001–2005. The use of patient

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samples was approved by the Helsinki Committee for Human Research of the Hadassah Hospital, Ein-Kerem, Jerusalem. DNA extracted from the cultured promastigotes was used for internal transcribed spacer 1 (ITS1) PCR,[16] and the products were further sequenced and used for phylogenetic analysis.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blotting. A stock of *L. tropica* parasites (MHOM/IL/2005/LRC-L1239) recovered from a patient in MA was used for antigen preparation and for sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blotting as previously described.[17,18] Blotted membranes were scanned using an Epson 3170 Photo Perfection scanner, (Visioneer, Pleasanton, CA) and the image was analyzed by a quantitative computerized technique as previously described.[17] Positive and negative controls of both hyraxes and humans were included in each assay.

Positive control sera were obtained from EI hyraxes (Nos. 1 and 2), and patients who were confirmed positive using ITS1 PCR for *L. tropica* infection. Negative control sera and DNA were obtained from healthy humans and 5 young hyraxes born in a non-endemic area and kept in captivity in a zoo.

DNA extraction. DNA was extracted from edetate calcium disodium anti-coagulated blood using the phenol-chloroform method.[19]

Internal transcribed spacer 1 polymerase chain reaction. The leishmanial ribosomal operon ITS1 region was amplified using the *Leishmania* specific primers LITSR and L5.8S essentially as previously described.[16] The reaction mixture was modified to contain dimethyl sulfoxide and high-fidelity DNA polymerase Phusion Hot Start (Finnzymes, Espoo, Finland) at final concentrations of 2.5% and 0.02 U per reaction, respectively, and was performed in a 25 µL total volume. The expected amplification product size was 314 bp.

Kinetoplast DNA real-time polymerase chain reaction conditions. Real-time PCR was performed using the Chromo 4 (Bio-Rad Laboratories, Inc. Hercules, CA) with the Dynamo HS, SYBR green qPCR kit (Finnzymes, Espoo, Finland). Primers JW11 (5′-CCTATTTTACACCAACCCCCAGT-3′) and JW12 (5′-GGGTAGG GGCGTTCTGCGAAA-3′) were used to amplify a 120 bp fragment from the kinetoplast minicircle as previously described.[20] To improve visualization, sets of melting peaks were derived from the first derivative of the fluorescence intensity versus temperature $-dI/dT$ (Intensity/temperature). Analysis was done using the Opticon Monitor™ software, 3.1.32 version.

Kinetoplast DNA real-time polymerase chain reaction sensitivity. Spiking of cultured *L. tropica* promastigotes into non-infected hyrax blood was made to quantify the number of parasites in naturally infected (NI) hyrax blood samples. *L. tropica* promastigotes (LRC-L1239) were grown, suspended in culture medium, and counted using a Coulter counter (Z2, Beckman Coulter, Inc., Brea, CA) to give a dilution of 1×10^4 parasites/mL. Ten µL of the above suspension were then mixed with non-infected hyrax blood to give serial dilutions of 10, 1, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} parasites/µL.

DNA was extracted from the above diluted samples using a DNA extraction kit (Promega Biosciences, Inc., Madison, WI). Dilutions were used to create a calibration curve determining the sensitivity of the assay as 0.01 parasites per 5 µL sample.

Phylogenetic analyses. Alignment of sequences was performed using Molecular Evolutionary Genetics Analysis software version 3.1. The data was analyzed using Phylogeny Inference Package software version 3.65 (http://evolution.genetics.washington.edu/phylip.html) applying the neighbor joining and maximum likelihood algorithms. Modeltest version 3.4[21] determined the Hasegawa-Kishino-Yano model (HKY85) to be the most appropriate substitution model for the database.[22,23] Further analysis was conducted using the heuristic search option with tree-bisection reconnection branch-swapping and 100 random trees.[24] Nonparametric bootstrap proportions were estimated from 1000 pseudo-replicate datasets. All characters were given equal weight during analysis. Nodes with bootstrap support less then 80% were collapsed.

RESULTS

Western blot. Sera from 36 hyraxes were tested by western blot for reaction with *L. tropica* antigen (Table 1). Reactions with 15 bands ranging from 17–92 kDa were observed. Of these bands, 8 (17, 20, 21, 23, 25, 30, 37, and 53 kDa) were also observed with sera from EI hyraxes and PCR-positive humans, suggesting that the appearance of antibodies against these antigens is indicative of exposure to *L. tropica* in both species. Reaction to each band was assessed according to its mean net intensity value using the Kodak 1D computerized program as previously described.[17] Twenty-nine of 36 hyraxes (80%) reacted with the *L. tropica* antigen by showing at least 1 of the 8 bands also detected by the human and EI hyrax positive controls. The remaining bands were not found to be useful for diagnostic purposes. Negative human and hyrax control sera were included in each assay and showed no reactivity with the *L. tropica* antigen.

Internal transcribed spacer 1 polymerase chain reaction. PCR amplification of the ITS1 region was performed on samples from 17 NI and 2 EI hyraxes, where sufficient DNA was available (Table 1). Four of 17 (23%) hyraxes from MA were positive (Table 1). The *L. tropica* ITS1 product from 3 NI hyraxes was sequenced and submitted to GenBank

Table 1
Hyraxes tested for *Leishmania tropica* by molecular and serologic methods

				Real-Time PCR	
Mode of infection*	Hyrax no.	Serology	ITS1 PCR	kDNA	Parasites/reaction
EI	Hyrax no. 1	+	+	+	25.8
EI	Hyrax no. 2	+	+	+	24
NI	424	+	+	+	0.04
NI	425	+	+	+	0.24
NI	426	+	–	–	–
NI	427	–	–	–	–
NI	428	–	–	+	1.6
NI	429	+	–	+	0.3
NI	430	+	–	+	2.2
NI	431	+	+	+	24.5
NI	432	–	–	–	–
NI	433	+	–	–	–
NI	434	+	–	–	–
NI	435	–	–	+	25.5
NI	436	–	–	–	–
NI	438	+	–	+	1.09
NI	439	–	–	–	–
NI	6827	+	–	+	0.34
NI	9055	–	+	+	0.3
Total (% positive)	17	10 (58)	4 (23)	10 (58)	

* NI = naturally infected; EI = experimentally infected.

(Accession numbers FJ595949; FJ595950; and FJ595951). Reference *L. tropica* promastigote samples ($N = 3$) isolated from *Ph. arabicus* and *Ph. sergenti* originating from NK as well as SK were also amplified by ITS1 PCR,[25] in addition to strains ($N = 11$) from humans in MA, SK, and NK regions.

Kinetoplast DNA real-time polymerase chain reaction. Real-time PCR for kDNA was performed on 17 NI and 2 EI hyrax blood samples (Table 1). The standard curve generated using hyrax blood spiked with parasites was linear over a 5-log range of DNA concentrations and had a correlation coefficient of 0.998. The reaction was sensitive down to a lower detection limit of 0.01 parasites per 5 μL sample, similar to previously reported parasite loads recorded for EI rodent blood.[20] The melting temperature of the amplicon was 80°C and identical for all products as determined by the melting curve. Ten of 17 NI hyraxes (58%) examined were found positive. Quantification of the samples (5 μL DNA) revealed a variety of parasite burdens ranging between 0.04–25.5 parasites per 5 μL sample (Table 1). Most of the hyraxes ($N = 8$) exhibited low parasite loads (0.04–2.2 parasites/5 μL sample) with the remaining animals including NI hyraxes and samples from EI hyraxes taken 8 weeks post infection showing 10-fold higher parasite loads. The kDNA real-time PCR products were sequenced and compared with sequences deposited in GenBank using BLAST hosted by National Center for Biotechnology Information, National Institutes of Health, USA (http://www.ncbi.nlm.nih.gov). The closest matches to the amplified products were always *L. tropica* kDNA sequences with an identity of ≥ 96% over the 109–111 bp amplified sequence.

Comparison of assays. Seventeen NI hyraxes were analyzed by all three assays. Ten of 17 hyraxes (58.8%) were positive by kDNA real-time PCR, 4/17 (23.5%) by ITS1 PCR, and 10/17 (58.8%) using western blotting. All samples positive by the ITS1 PCR were also positive by kDNA real-time PCR. Out of the 17 samples, 7 (41.2%) were positive by PCR and serology. Three samples were positive by serology with no molecular evidence of infection.

Despite the fact that 8/10 of the kDNA PCR positive samples had low parasite loads, 6/8 (75%) of these animals gave positive reactions in western blot analysis. Correlation coefficient comparing between the different assays was calculated using Cohen's kappa test and indicated a positive association of 0.6 between western blotting and kDNA real-time PCR.

Phylogenetic analysis. The strains of *L. tropica* used for analysis are listed in Table 2 and include human, hyrax, and sand fly strains from three locations: MA, NK, and SK. The size of amplified fragments was ~241–300 bases of which a 241 bp fragment was selected as suitable for analysis. Multiple alignment showed 187/241 conserved sites, 39/241 variable sites, and 26/241 parsimony informative sites. Topology for the ITS1 fragment obtained from maximum likelihood and neighbor joining analyses carried out on the database (241 nucleotides) is shown in Figure 1. The ITS1 phylogram demonstrates clustering of *L. tropica* strains from NK as one group, supported by strong bootstrap values of 100% independently by the two analytic methods used while denoting SK together with MA as a separate group. This grouping is supported by a 3 bp difference (T insertion and GG deletion) found in the NK strains. According to both methods, the MA and SK groups appear as sister populations including strains from humans as well as sand flies and hyraxes, which were denoted as phylogenetically similar.

TABLE 2

Isolates of *Leishmania tropica* used for phylogenetic analysis

Source/WHO code*	Origin	Species
Hyrax 424 (GenBank accession FJ595949)	MA	Hyrax
Hyrax 425 (GenBank accession FJ595950)	MA	Hyrax
Hyrax 9055 (GenBank accession FJ595951)	MA	Hyrax
MHOM/IL/2005/LRC-L1186	MA	Human
MHOM/IL/2005/LRC-L1217	MA	Human
MHOM/IL/2005/LRC-L1221	MA	Human
MHOM/IL/2005/LRC-L1224	MA	Human
MHOM/IL/2005/LRC-L1226	MA	Human
MHOM/IL/2005/LRC-L1241	MA	Human
MHOM/IL/2005/LRC-L1243	MA	Human
ISER/IL/2004/LRC-L1155	SK (Tiberias)	Sand fly
MHOM/IL/2001/LRC-L838	SK (Tiberias)	Human
MHOM/IL/2001/LRC-L837	SK (Tiberias)	Human
MHOM/IL/2001/LRC-L836	SK (Tiberias)	Human
MPRO/IL/2003/Hyrax107	NK (Amnun)	Hyrax
MHOM/IL/2002/LRC-L863	NK (Karkom)	Human
ISER/IL/2002/LRC-L909	NK (Karkom)	Sand fly
IARA/IL/2002/LRC-L910	NK (Amnun)	Sand fly

MA = Maale-Adumim; NK = North Kinneret; SK = South Kinneret.
*World Health Organization (WHO) code

DISCUSSION

Although CL caused by *L. tropica* is usually considered an anthroponotic infection,[15] in Israel, Jordan, and the Palestinian Authority, it appears to be a zoonosis.[3,4] The present study reveals a high rate of *L. tropica* infection in hyraxes from the human focus of CL in MA and the presence of the parasite DNA in the blood of this suspected reservoir animal.

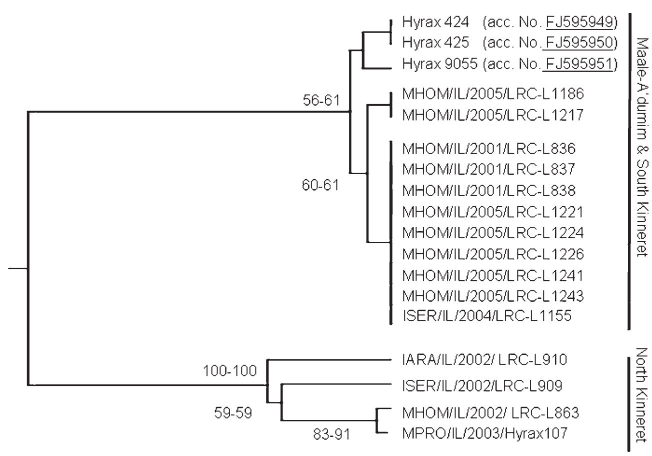

FIGURE 1. ITS1 sequence phylogram of *Leishmania tropica* strains obtained by the Tree Branching Rearrangement option of the Phylogeny Inference Package (PHYLIP) software, version 3.65, (http://evolution.genetics.washington.edu/phylip.html) performed on the complete database obtained (241 nucleotides). *Leishmania major* was used as an out-group. The same database created two identical trees using the Maximum Likelihood and Neighbor Joining algorithms with bootstrap values for both algorithms (after 1000 replicates) indicated on the branches, respectively. GenBank accession numbers of *L. tropica* from hyraxes submitted from this study are shown in brackets. Numbers on nodes represent similarity. MHOM, human; IARA, *Phlebotomus arabicus* sand fly; ISER, *Ph. sergenti* sand fly; MPRO, rock hyrax (*Procavia capensis*); acc. No.: GenBank accession number.

This is the first study to perform quantitative PCR, as well as serology, for the detection of leishmanial infection in hyraxes. Previous studies in northern and central Israeli *L. tropica* rural foci showed that 10–13% of the hyraxes tested by ITS1 PCR were positive for parasite DNA.[7,25] This is approximately half the value found (23.5%) in this study using the same assay. In addition to the ITS1 PCR, we used a highly sensitive kDNA real-time PCR to obtain a more accurate value for the prevalence of *L. tropica* infection in hyraxes and compared it with ITS1 PCR. Conventional kDNA PCR targeting minicircles is approximately 10–100-fold more sensitive than reactions targeting the ITS1 region, and real-time PCR for *Leishmania* has been shown to be more sensitive than conventional PCR assays.[26–28] In the current study, hyrax blood samples examined using kDNA real-time PCR revealed an extremely high infection rate of 58.8% in the MA urban disease focus in central Israel.

The hyrax samples evaluated in previous studies included snout, ear pinna, and lymph node biopsies taken from both naturally and experimentally infected animals.[7,12] In other studies, laboratory diagnosis of infection was achieved mostly by testing dermal biopsies, both in humans and in hyraxes.[7,25,29] The presence of *L. tropica* DNA in hyrax blood shown in the current study could be due to the circulation of live amastigotes present in phagocytes or to degraded parasites. *Leishmania tropica* has been shown to cause a visceralizing infection in humans and dogs,[30–32] however the possibility of infection involving visceral organs and circulatory blood dissemination in the hyrax requires substantiation in further studies.

The diverse parasite loads found in hyraxes in this study could be due to the variable time course from infection, variation in the immune response between individual hyraxes, or due to strain virulence. The hyrax samples were collected in March, which is at the end of the winter, several months after the end of the sand fly activity season in Israel. Three of the hyraxes that were positive by real-time PCR were negative by serology and conversely, three different hyraxes that were negative by serology were positive by real-time PCR. A possible reason for hyraxes being PCR-positive and seronegative could be due to the absence of an antibody response despite infection as found for dogs and humans with *L. infantum* infection.[33–35] The opposite situation with seropositive and PCR-negative hyraxes could be due to infection that has resolved with a persisting antibody response, or due to the absence of parasite DNA from the blood despite infection in the skin. Cross reactivity with other pathogens such as *Trypanosoma* spp. is possible, but unlikely, because no infection with trypanosomes has been described in hyraxes in Israel. In addition, trypanosomiasis is extremely rare in Israel with an isolated report of *Trypanosoma evansi* describing sporadic infection in horses and camels in the Arava region in southern Israel.[36]

Phylogenetic analysis showed that ITS1 DNA sequences of *L. tropica* parasites from human CL and hyraxes in MA were highly similar, thus further incriminating the vector and reservoir hosts of *L. tropica* in this focus. The genetic divergence of *L. tropica* into the NK and MA clades, as found in this study, supports previous findings of discrete *L. tropica* genotypes in these areas differing in vector competence and biochemical characteristics.[25]

Western blot revealed that 80% of the hyraxes had detectable antibody responses to *L. tropica*. Although serological studies of antibody responses have not been reported previously with hyraxes, it has been used for the diagnosis and study of *L. tropica* CL.[29,37] Several of the antigens, 17, 20, 21, 23, 25, 30, 37, and 53 kDa, recognized both by infected hyrax and human serum show molecular weights similar to those used in the serodiagnosis of *L. tropica* CL (27 and 30 kDa), or for monitoring treatment (19 kDa).[37] Thus, hyrax antibody reactivity to specific *L. tropica* antigens resembles the response mounted by infected humans. Variability in the western blot technique could account for slight protein migration differences. Western blot with human sera was found to be more sensitive and valid than the conventional enzyme-linked immunosorbent assay for the serodiagnosis of CL, and it was shown to be useful as an alternative diagnostic tool in instances when it was difficult to demonstrate the presence of parasites in stained tissue smears.[37]

Serology is considered an indicator of exposure rather than infection. The discrepancy between the rates of infection shown by PCR and exposure demonstrated by serology in this study could be explained by past infection with persisting antibody levels, possible presence of infection without detectable parasite DNA in blood, or cross-reactivity with trypanosomes or other closely related organisms. However, infection of hyrax with trypanosomes or other known cross-reactive agents has not been reported to date.[38] As expected, serological results were in moderate (60%) statistical agreement with real-time PCR. Western blotting served as a parameter for exposure to *L. tropica* antigen, while real-time PCR indicates active infection by monitoring parasite DNA. These two diagnostic approaches complement each other in evaluating the incidence of hyrax infection in human CL foci caused by *L. tropica*.

In conclusion, the high rate of hyrax infection with *L. tropica* in the CL focus of MA and the similarity between local human and hyrax parasite strains strongly supports the role of the hyrax as a reservoir for human disease in this urban focus. Efforts to prevent human disease should focus on interrupting the transmission of infection involving hyraxes and humans.

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